Exhibit A:

MANUSCRIPT SHOWING CARDIO-PROTECTIVE EFFECTS OF A FATTY ACID FORMULATION SIMILAR TO CLAIMED COMPOSITION (SUBMITTED FOR PUBLICATION IN 2009)

Modulation of myocardial resistance to ischemia-reperfusion injury by dietary fatty acids.
Insights into the concept of cardioprotection by Mediterranean diet

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Short title: Mediterranean diet and cardioprotection.

ABSTRACT

Zeghichi-Hamri S, de Lorgeril M, Salen P, Laporte F, Chibane M, Boucher F, de Leiris J. Modulation of myocardial resistance to ischemia-reperfusion injury by dietary fatty acids. Insights into the concept of cardioprotection by Mediterranean diet. *Am J Physiol Heart Circ Physiol 2009.* --

Although the Mediterranean diet (MED) is considered the optimal diet to prevent coronary heart disease (CHD), it is still unknown whether adoption of MED may result in improved myocardial resistance to ischemia-reperfusion injury. We now investigated whether a diet low in saturated fats and omega-6 fatty acids (ω6) but rich in plant and marine omega-3 fatty acids (ω3), a typical MED fatty acid profile, may result in smaller infarct size and better left ventricular function (LVF) recovery in a rat model of regional ischemia-reperfusion. The MED rats were compared with rats receiving diets high in either saturated or ω6 fatty acids. The effects of the three diets were examined by analyzing the fatty acid composition of plasma, erythrocyte cell membranes and phospholipids of myocardial mitochondria. Results demonstrate a great accumulation of ω3 and a parallel decrease of arachidonic acid (the main ω6) in plasma, cell membranes and cardiac mitochondria. Also, the MED rats developed smaller infarct size compared with the control groups ($p<0.01$) while short-term LVF recovery was not different in the three groups. Data suggest that plant ω3 might be critical in that protection by lowering arachidonic acid. In conclusion, MED-induced cardioprotection may be partly related to an improved resistance to myocardial ischemia-reperfusion injury.

INTRODUCTION

Dietary fats play an important role in coronary heart disease (CHD) (5,9,10, 17). Beyond their well-known effects on atherosclerosis, thrombosis and the risk of cardiac death (5,9,10,17), it is still unclear whether specific dietary fatty acid profiles modulate the myocardial resistance to ischemia and reperfusion injury. This is a critical issue because myocardial resistance to ischemia-reperfusion is a major determinant of myocardial infarct size which in turn is a causal factor in the development of CHD complications such as cardiac pump failure and malignant ventricular arrhythmias. For instance, it is still unknown whether dietary fatty acid profiles associated with the Mediterranean diets (9,10) result in smaller infarct size after regional ischemia-reperfusion. As the Mediterranean diet (MED) was shown to be very protective against CHD complications (9,10,29,45), it is important to understand by which mechanism(s) MED is protective. There are several fat and non fat components in MED (9,10,29,45). It is, however, believed that typical MED dietary fatty acid profiles might be critical in the MED-induced cardioprotection (9,10). Depending of the geographic area, there are several MED fatty acid profiles. The most common one is low in both animal and plant saturated fats, low in *trans* fatty acids and plant omega-6 fatty acids (ω6) but rich in both plant and marine omega-3 fatty acids (ω3) (9,10).

The main aim of the present study was therefore to investigate the effect of that specific MED fatty acid profiles on both infarct size and left ventricular function recovery in a rat model of regional ischemia-reperfusion. For that purpose, we used an isolated heart model which allows studying the response of the myocardium itself, independent from other organs, from neurological brain-heart connections and from blood components (*biological milieu*) such as circulating cells, platelets, hormones or cytokines which are all potentially influenced by dietary fats. Although that *biological milieu* is influenced by dietary fats and can by itself influence the myocardial response to ischemia-reperfusion, the isolated working heart model was preferred to an in vivo model in order to specifically study the myocardial response independently from the *biological milieu*.

To evaluate the effects of MED, we used two comparison groups: the first one received palm oil (rich in saturated fats but poor in ω6 and ω3 and named PO) and the second one received sunflower oil (low in saturated fats and ω3 but extremely rich in ω6 and named

SO) in addition to the usual low fat chow diet. We thus compared three diets with similar energy and fat intake but very different fatty acid profiles. The effects of the three diets were examined by analyzing the fatty acid composition of plasma, erythrocyte cell membranes and phospholipids of cardiac mitochondria. We used erythrocytes because of their short half-life (compatible with dietary protocols in animals) and because they are known to reflect the fatty acid composition of cardiac cell membrane (24). We evaluated the fatty acid composition of the main phospholipids of cardiac mitochondria because mitochondria phospholipids are thought to play a central role in the myocardial resistance to ischemia-reperfusion (8,22,32,38,46).

MATERIALS AND METHODS

This study conforms to the *Guide for the Care and Use of Laboratory Animals,* National Academic Press, Washington, DC, 1996. Male Wistar rats (IFFA-Credo, L'Abresle, France) were used throughout this investigation. All animals received a basic laboratory solid low fat chow diet (regime A04, UAR, Villemoisson-sur-Orge, France) and water ad libitum. All rats (n=48 for blood and cardiac lipid measurements and n=48 for cardiac experiments) were fed dietary supplements by gavages during 8 weeks daily at the same hour of the day. Animals were randomly divided into three groups according to their dietary supplements. Animals in the palm oil group (PO) were supplemented with 650 µl palm fat (Palmella, Germany), rich in saturated fatty acids but low in ω6 and ω3. Animals in the sunflower group (SO) were supplemented with 650 µl Sunflower oil (Lesieur Inc, France) poor in saturated and ω3 but rich in ω6 and those in the MED group were supplemented with 650 µl of a mix of plant and marine ω3 (*Mixalpha*, Synergia, Beaune-sur-Arzon, France). Mixalpha is a mixture of linseed and fish oils. The fatty acid composition of the palm, sunflower and Mixalpha oils is shown in Table 1. The dose of 600 mg *Mixalpha* was calculated to bring the final diet of the MED group to a ratio of 18:2ω6/18:3ω3 close to 1, the optimal ratio for adequate conversion of 18:3ω3 to the very long-chain ω3 and to decrease the conversion of 18:2ω6 in arachidonic acid. The food intake in each group was checked every day, animals were weighed once a

week and housed under conditions of constant temperature, humidity and standard light-dark cycle.

Cardiac experiments

Heart preparation and perfusion were carried out according to methods described (20,21,44). Briefly, rats were anaesthetized with pentobarbital sodium (Sanofi, 40 mg/kg, i.p.) and heparinized (Sigma; 100 UI/rat, i.v.). Hearts were excised, washed in cold Krebs-Henseleit buffer, suspended and canulated via the aorta and perfused at a constant pressure of 9.81 kPa (1 m H_2O) using the Langendorff mode with Krebs-Henseleit crystalloid buffer (20,21,44) (containing in mM: NaCl 118; KCl 4.75, $NaHCO_3$ 25, $MgSO_4.7\ H_2O$ 1.19, KH_2PO_4 1.18, $CaCl_2.H_2O$ 1.36 and glucose 11.1) and equilibrated with a mixture of O_2/CO_2 (95%/5%) at 37°C, pH 7.4. Then, after removing sinus node, the heart was paced at 5 Hz (300 bpm) via a monopolar electrode placed on the left atrial wall, connected to a stimulator (6021 SRI, UK). Left ventricular (LV) pressure was measured with a transducer (Statham P23ID, Gould, France) connected to a noncompliant water-filled ultra thin balloon introduced into the LV cavity with a volume adjusted to preset a baseline end-diastolic pressure of 4 mmHg (20,21,44). A 5-0 silk snare was passed under the left-coronary artery close to its origin. After 15-min equilibration period and normoxic perfusion, the left coronary artery was occluded by tightening the snare for 30 minutes and then reperfused for 120 minutes. All hearts were kept at 37° C in a thermostatically controlled glass chamber throughout the experiment protocol. For each heart, coronary flow was measured and myocardial function was recorded after 15-min stabilization and then every 10 minutes. After 120 minutes of reperfusion and retightening of the coronary snare, a solution of Evans Blue was injected through the aorta to delineate the nonstained risk zone (RZ). The hearts were then briefly frozen in liquid nitrogen and stored at –20°C. They were then cut into 6 to 7 transverse slices of 1mm thickness. Slices were incubated in 1% triphenyltetrazolium chloride in sodium phosphate buffer at 37° C during 20 minutes to stain viable cells in the risk zone. Volume of infarct and risk zone was calculated using image software (NIH AutoExtractor 1.51). Risk zone was expressed as percent of total ventricular volume and infarct size as percent of risk zone.

Cardiac mitochondria were isolated as described (39). Briefly, cardiac tissue was minced, washed, suspended in 10ml of an isolation medium, subjected to mild trypsin digestion for 30 min at 4°C, and then diluted in the isolation medium (23) containing BSA and trypsin inhibitor. Homogenate was centrifuged for 10 min at 600g and the supernatant was decanted and centrifuged at 8000g for 15min to obtain the mitochondrial pellet. The upper layer was discarded and the tightly packed dark pellet was resuspended and washed 3 times. Mitochondria were purified on a discontinuous gradient consisting of 6% Percoll, 17% and 35% metrizamide (42). After 30 min centrifugation at 50000g, a narrow band was recuperated to sediment mitochondria for 10 min at 8000g.

Fatty acid analyses

Plasma lipids and erythrocyte phospholipids were extracted in hexane/isopropanol (23). Fatty acids were first methylated and after extraction with hexane, methyl esters were separated and quantified by gas chromatography (Hewlett Packard 5890 Series Gas Chromatography). Methyl ester peaks were identified by comparing their retention times to those of a standard mixture. Each fatty acid was expressed as percent of total fatty acids. Mitochondrial lipids were extracted in hexane/isopropanol and dissolved in chloroform/methanol containing cholesterol acetate as an internal standard. Total phospholipids, neutral lipids and internal standard were separated by thin layer chromatography followed by flame-ionization detection in Iatroscan TLC-FID (Iatron laboratory, Tokyo Japan). Phospholipids concentration was expressed as percentage of total phospholipids.

After lipid extraction, mitochondrial phospholipids were separated from the total lipid extract by thin layer chromatography on silica gel plates with chloroform/methanol/water as solvent. PC, PE and CL spots were scraped directly into test tubes, dissolved in chloroform/methanol, evaporated under N_2 and mixed with 0.1mg/ml heptadecanoïc acid (6). Then, the same method used for blood fatty acids analysis was applied.

Statistics

Data are expressed as mean ± SEM. Measurements were analyzed by ANOVA with between group differences tested by post-hoc application of Tukey's test. For all tests, $p<0.05$ was considered significant.

RESULTS

After 8-week supplementation, the SO group was slightly heavier compared with the PO and MED groups although the differences did not achieve statistical significance (Fig. 1). In the lipid measurement experiments, two animals in the SO group were excluded from the analysis of cardiac phospholipids due to technical problems. In the cardiac experiment, nine animals were lost during either the gavages' period or at the moment of the ischemia-reperfusion experiments (technical difficulties). Finally, five animals were excluded because of total absence of ischemia (lack of ischemic zone) leaving 13, 10 and 11 rats in the PO, SO and MED groups respectively for the analyses of infarct size and LV function.

As shown in Table 2, there was no difference between the 3 groups for standard food and total fat intakes. The main differences between groups regarded saturated, ω3 and ω6. Blood lipids and plasma fatty acids are shown in Table 3. Total and HDL cholesterol were significantly lower in the MED group while triglycerides were not significantly different. Regarding plasma fatty acids, the main differences between groups were for total ω6 ($p<0.0001$) and arachidonic acid (20:4ω6 $p<0.0001$) which were strikingly lower in the MED group. Total plasma ω3 and each individual ω3 were strikingly higher in the MED group ($p<0.0001$).

Erythrocyte fatty acid composition is shown in Table 4. There was no significant difference between groups for total saturated and total polyunsaturated fatty acids. The main difference between groups regarded total ω6 and total ω3 ($p<0.0001$). In addition, in the MED group, 20:4ω6 was remarkably lower ($p<0.0001$) and 20:5ω3 higher ($p<0.0001$) compared to the PO and SO groups giving a ratio 20:4ω6/20:5ω3 of 66, 127 and 4.5 respectively for the PO, SO and MED groups respectively ($p<0.0001$).

Table 5 shows the fatty acid composition of the 3 main phospholipids of cardiac mitochondria. The main differences between groups were for total ω3 and for each ω3 (which were consistently higher in the MED group), and for 20:4ω6 which was, apart from cardiolipin, lowered in MED compared to PO and SO. In phosphatidylcholine and phosphatidylethanolamine, the main differences between groups were for total and each

individual ω3 and for 20:4ω6. In cardiolipin, saturated fatty acids were consistently higher in PO.

Cardiac experiments

At baseline and throughout the ischemia-reperfusion period, there was no significant difference between groups for hemodynamics. In particular, left ventricular function and coronary flow at baseline (normoxic perfusion) were not different between groups (Table 6, A). Likewise, after 30-min and 120-min reperfusion (Tables 6, B and C), we found no significant difference between groups for coronary flow, diastolic pressure and LVDevP. The risk zone was not different in the three groups (Fig. 2). In contrast, infarct size was significantly different between groups, 37.7±2.1, 32.9±1.5 and 28.6±1.7 % of risk zone in the PO, SO and MED groups respectively (ANOVA $p<0.01$). Compared with either PO or SO, MED reduced infarct size by 24.1% and 13.1% respectively ($p<0.05$). When PO and SO (the two groups with low ω3) were pooled together and compared with MED (with high ω3), infarct size was still significantly smaller ($p<0.05$) with 35.3±1.4 for PO+SO and 28.6±1.7 for MED. In contrast, when PO and MED (the two groups with low ω6) were pooled together and compared with SO (with high ω6), there was no significant difference.

DISCUSSION

This is the first study showing that one of the most common MED fatty acid profile results in a relative resistance to myocardial ischemia-reperfusion injury. Thus, part of the protection resulting from the adoption of a traditional MED in humans might be due to smaller infarct size following ischemia-reperfusion. Whatever the precise molecular mechanism, this kind of relative myocardial resistance looks like the "ethanol preconditioning" (20,21) and the flavonoid-induced state of myocardial resistance to ischemia-reperfusion injury (44) that we recently described. Although both moderate ethanol drinking and high flavonoid intake are major components of the traditional MED, it does not mean that MED is protective only through the induction of that relative resistance to ischemia-reperfusion injury. Actually, there are other pathways

(haemostasis, for instance) through which lipid or non lipid MED factors could protect against CHD complications (9,10,29,45).

Clinical implication

In the present study, we have examined the effects of a MED fatty acid profile with high ω3 intake associated with low intakes in saturated fatty acids and ω6, and in the absence of *trans* fatty acids. It means that the observed protection against ischemia-reperfusion likely resulted from interplay between several lipid factors and not from a unique nutrient. This is a common problem encountered in both experimental and clinical nutrition because any change in the diets of experimental animals or humans is multi-factorial. When studying diets with identical energy intake, reduction of one nutrient results in a proportional increase in one or several other nutrients (12). It is often difficult to decide whether one individual factor is more active than the others or whether one particular change is more important than the others.

The next issue is therefore to examine whether the beneficial effect of that MED fatty acid profile is predominantly due to one of this component. For instance, as shown on Figure 2, the hearts of rats with high saturated fat intake were the less resistant compared with the two other groups. On the other hand, this study shows that for the same amounts of fat, the hearts of rats receiving ω3 are relatively more resistant to ischemia-reperfusion injury compared with those receiving ω6. This is probably of clinical relevance because high ω6 intake has been encouraged for many years to replace saturated fats in the context of cholesterol-lowering diets to prevent CHD in humans (11). This study shows that the ω6-rich dietary strategy is not optimal, at least in terms of myocardial resistance to ischemia-reperfusion, and actually confirms clinical trials which suggested no beneficial effect of ω6-rich diets (11). Thus, the present study indicates that to help the heart to resist to ischemia-reperfusion injury, one dietary strategy would be to decrease both saturated fats and ω6 and to increase ω3, as also suggested by clinical trials (9,10,29,45).

The potential importance of ω3 in myocardial resistance to ischemia-reperfusion

Previous experimental studies have been conducted to evaluate the effects of specific dietary fatty acids on the ischemic myocardium and their ability to prevent myocardial

complications (2,3,7,13,14,16,27,30,31,33,36,37,38,43,47,48). Most of these studies, however, did not specifically study myocardial resistance to regional ischemia-reperfusion, rather the effects of dietary fats on global ischemia-reperfusion and ventricular arrhythmias (3,14,30,31,33,43,48). In addition, most of them suffer from some methodological weaknesses, for instance too short ischemia to induce histological cell necrosis (27,38,43,48). In other studies, there was no clear definition of the comparison groups in terms of dietary fatty acid profile and the biochemical changes induced by the tested diets in cell membranes and specifically in the myocardium were not properly evaluated (7,27,36,47). Finally, most studies focused on very-long chain (marine) ω3 or fish oil (and not plant ω3) with the main objective to show that they reduce the risk of ventricular arrhythmias. Only few groups actually tested the myocardial resistance to regional ischemia-reperfusion (16,37,43) although it is the best experimental model of myocardial infarction. For instance, Oskarsson et al have shown that marine ω3 reduce infarct size in a canine model of ischemia-reperfusion (37). In that study, however, the total amounts of fats were not similar in the experimental and control groups leaving the possibility that the smaller infarct size was not the result of a protection specifically induced by marine ω3. Actually, in the main study of transient regional ischemia-reperfusion with appropriate control groups using the isolated working heart model, marine ω3 did not reduce infarct size (16). In that study, fats were given in the control groups under the form of corn oil (rich in ω6 but poor in saturated fatty acids and ω3) or beef tallow (rich in saturated fats but poor in ω6 and ω3). Thus, the dietary protocol was quite similar as the one used in our present study but the results were different since our MED rats were relatively protected (about 20% reduction of infarct size) whereas there was no effect of marine ω3 in the study of Force et al (16). The main difference between the two studies is that we gave both plant and marine ω3 to our rats whereas Force et al were given marine ω3 only (16). This raises the possibility that alpha-linolenic acid (ALA), the main plant ω3, was the protective factor in our study.

Few studies have suggested that ALA may have a direct protective effect on the heart (2,15) or the brain (25), and to our knowledge no study examined the specific effect of

ALA on regional ischemia-reperfusion and infarct size. In our study, ALA was considerably higher in the plasma, erythrocyte membranes and mitochondrial phospholipids of the MED rats compared with the PO and SO rats but the relative contribution of ALA to total fatty acid remained quite small (Tables 4 and 5). In a previous pilot study, we did not observe protection with ALA-rich oils. Thus, the protective role of ALA by itself (given without marine ω3) remains speculative. It is noteworthy that in an animal model of hereditary cardiomyopathy, ALA supplementation resulted in preservation of myocardial structure and function (15). This study, however, did not investigate the effect of ALA in the context of ischemia-reperfusion.

One possibility would be that ALA is cardioprotective against regional ischemia-reperfusion injury when it is associated with marine ω3, or when given in the context of MED. A third possibility is that ALA supplementation was protective by two mechanisms: by itself and through induction of a massive reduction of the concentrations of arachidonic acid (AA), the main ω6 in cell membrane and mitochondrial phospholipids. Despite higher dietary AA intake compared with the PO group and similar levels of linoleic acid (C18:2ω6, the precursor of AA), the MED rats had considerably lower AA levels (30 to 50% lower levels) compared with PO and SO. This suggests that ALA supplementation induced a strong inhibition of the endogenous synthesis of AA from its precursor linoleic acid, a result that was not unexpected (4,34,41). It could be argued that supplementation in marine ω3 (as in the study by Force et al), also results in decreased AA. However, the decrease in AA following a diet rich in both marine ω3 and ALA is much more important than after supplementation in marine ω3 only. In a parallel study (data not shown) where only marine ω3 were given to rats, the decrease in plasma AA reached 23% (against 51% with the MED fatty acids) and the ratio AA/EPA decreased by 75% only against 96% with the MED fatty acids. The next question is whether such a decrease in AA levels may explain, at least partly, the protection observed in our study and the lack of protection in the study by Force et al using similar isolated heart model and comparable control groups (6). AA is often presented as a major player in CHD complications (26) and likely responsible of cell damage after ischemia-reperfusion (35). A massive accumulation of AA and lipoxygenase and cytochrome P450

epoxygenase metabolites has been reported in the post-ischemic myocardium (1,19,40). However, beside the production of well-characterized detrimental AA-metabolites, production of protective end products of AA metabolism has also been reported (18). Thus, from these previous and our own data, it is difficult to reach a conclusion. Further studies using complex experimental models to examine the biological effects of the many and various AA metabolites on the heart are therefore required to evaluate whether decreased AA levels in our model of ischemia-reperfusion actually result in better resistance to regional ischemia-reperfusion.

MED fatty acid profile and hemodynamics

We didn't find any difference in LVDevP and diastolic pressure as well as in coronary flow between the three groups before and after ischemia-reperfusion. It is indeed quite surprising that the limitation of infarct size (although modest) was not associated with improvement in ventricular function. It is well known, however, that brief periods of ischemia can have both negative effect on ventricular function and a powerful protective effect against cell necrosis (28). Thus, in our study, the chronically preconditioned hearts might have developed a kind of myocardial stunning that could have masked the better recovery of post-ischemic function compared to the experimental groups. On the other hand, if the isolated heart model is a good model to study acute regional myocardial response to ischemia-reperfusion (infarct size) independently from the *biological milieu*, it is likely not the good one to examine long-term post-ischemic LV function recovery. Post infarction recovery would be more consistently determined 2 to 7 days following the acute ischemic episode. Also, an in vivo model would have been more appropriate for that purpose. Further studies using such a more appropriate model are needed to study the effect of MED dietary fatty acid profile on long-term LV function recovery.

Limitations of the study

One limitation of that study is the lack of explanation regarding the mechanisms of the relative myocardial resistance to ischemia-injury induced by the MED fatty acid profile. Since this study was not designed to specifically explore the mechanisms through which any specific fatty acid combination induces resistance to ischemia-reperfusion injury, we can only speculate on the beneficial effects of the association of plant and marine ω3. Also, from these data, it is difficult to separate the effects of reducing saturated and ω6

fatty acids and the effects of increasing plant and marine ω3. Finally, as discussed above, further studies using complex protocols are needed to decide whether striking reduction of AA in the context of MED may be protective.

This study actually suggests that MED induces a kind of chronic "preconditioning state". That relative protection is indeed observed when using an isolated heart system model with exclusion of all extra-cardiac factors potentially influenced by dietary fats and potentially playing a role in the induction of a chronic resistant state. This relative protection therefore was likely largely dependent on changes to the cardiac tissue itself. However, we cannot rule out the possibility that modification in the vasculature of the heart for instance may be involved in the protection. It is also possible that these lipid factors were not directly responsible for the protection but did induce generation of another signalling molecule that itself is responsible for the beneficial action. Further studies are required in different animal models to confirm our data and identify the molecular signaling pathway involved in the observed protection.

We conclude that a MED fatty acid profile, with its plant and marine ω3 components, results in a relative cardiac resistance to ischemia-reperfusion. From a clinical point a view, this is a confirmation that the Mediterranean diet appears to be the optimal diet to reduce CHD complications including those resulting from myocardial injury.

LEGENDS OF THE FIGURES

Figure 1: Effect of 8-week supplementation on body weight in the 3 groups

Figure 2: Risk zone (% total LV volume) and infarct size (% risk zone) in the 3 experimental groups. ANOVA P: non significant for risk zone and $p<0.01$ for infarct size.

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Table 1: Fatty acid composition of palm (PO), sunflower (SO) and Mixalpha oils (expressed as % of total fatty acids).

Fatty acids	PO	SO	Mixalpha
Saturated			
C14:0	1.1	-	0.1
C16:0	49.5	5.8	3.9
C18:0	4.9	4.7	1.9
Monounsaturated			
C18:1ω9	35.0	27.5	9.5
C16:1ω7	0.2	0.1	0.3
C18:1ω7	0.6	0.5	0.6
Polyunsaturated			
Total ω6	8.1	60.6	12.3
C18:2ω6	8.1	59.6	11.4
C20:4ω6	-	0.8	0.4
Total ω3	0.1	0.2	71.3
C18:3ω3	0.1	0.1	47.4
C20:5ω3	-	0.1	7.4
C22:5ω3	-	-	1.5
C22:6ω3	-	-	15.0

Table 2: Fat intakes in the 3 groups

	PO (n=16)	**SO** (n=16)	**MED** (n=16)	**P**
Standard food (mg/day/rat)	630 ± 11.7	696±6.9	603 ± 10.5	NS
Fat supplement (mg/day/rat)	600	600	600	-
Total fats (% total energy)	17.33 ± 0.1	17.02±0.1	17.25 ± 0.1	NS
Saturated (mg/day/rat)	464.6 ± 2.4[1]	208.0±1.4	159.5 ± 2.1	<0.0001
Polyunsaturated (mg/day/rat)				
Total ω6	374.3 ± 6.1	723.4±3.6[2]	386.8 ± 5.4	<0.0001
C18:2ω6	371.2 ± 6.0	713.5±3.5	378.4 ± 5.4	<0.0001
C20:4ω6	1.9 ± 0.0[3]	6.5±0.0	4.2 ± 0.0	<0.0001
Total ω3	44.0 ± 0.8	49.2±0.5	469.6 ± 0.7[4]	<0.0001
C18:3ω3	24.5 ± 0.4	27.7±0.3	307.4 ± 0.4[4]	<0.0001
C20:5ω3	6.9 ± 0.1	8.2± 0.1	51.1 ± 0.1[4]	<0.0001
C22:5ω3	1.3 ± 0.1	1.6±0.1	10.2 ± 0.1[4]	<0.0001
C22:6ω3	11.3 ± 0.2	12.5±0.1	100.9 ± 0.2[4]	<0.0001

Means ± SEM. [1] p<0.0001 vs. SO and MED, [2] p<0.0001 vs. PO and MED, [3] p<0.0001 vs. SO and MED, [4] p<0.0001 vs. PO and SO.

Table 3: Blood lipids (g/L) and plasma fatty acids (as % of total fatty acids)

	PO (n=16)	SO (n=16)	MED (n=16)	P
Blood lipids				
Total cholesterol	0.53±0.04	0.56±0.03	0.41±0.02	0.001
HDL cholesterol	0.30±0.01	0.33±0.01	0.25±0.01	0.001
Triglycerides	1.50±0.20	1.36±0.25	1.00±0.06	0.13
Plasma saturated fatty acids				
C14:0	0.81±0.05	0.70±0.05	0.68±0.04	0.11
C16:0	21.7±0.46[1]	19.3±0.39	19.3±0.58	0.001
C18:0	5.73±0.26	6.19±0.19	5.62±0.19	0.11
Plasma polyunsaturated fatty acids				
Total ω6	42.7±1.15	49.3±1.34	36.2±0.89[2]	<0.0001
C18:2ω6	26.2±0.78	28.2±0.66[3]	25.8±0.61	0.03
C20:4ω6	14.9±0.91	19.1±0.95	9.32±0.41[2]	<0.0001
Total ω3	5.90±0.17	4.84±0.14	21.0±0.71[2]	<0.0001
C18:3ω3	1.08±0.09	0.97±0.03	4.40±0.15[2]	<0.0001
C20:5ω3	0.74±0.03	0.55±0.02	6.95±0.36[2]	<0.0001
C22:5ω3	0.55±0.03	0.52±0.02	2.37±0.10[2]	<0.0001
C22:6ω3	3.22±0.13	2.57±0.12	6.98±0.28[2]	<0.0001

Mean ± SEM; [1] $p<0.01$ vs. SO and MED; [2] $p<0.0001$ vs. PO and SO; [3] $p<0.05$ vs. PO and MED.

Table 4: Erythrocyte fatty acids (as % of total fatty acids) in the 3 groups

	PO (n=16)	SO (n=16)	MED (n=16)	P
Saturated				
C14:0	0.23±0.01	0.25±0.02	0.24±0.01	0.80
C16:0	25.1±0.33	24.0±0.23	24.7±0.35	0.10
C18:0	11.6±0.38	12.9±0.31	11.6±0.45	0.07
Polyunsaturated				
Total ω6	45.7±0.51	47.7±0.44	36.8±0.44[1]	<0.0001
C18:2ω6	11.8±0.25	12.3±0.25	12.7±0.34	0.08
C20:4ω6	31.0±0.47	31.9±0.35	22.4±0.39[1]	<0.0001
Total ω3	6.37±0.17	4.83±0.13	15.9±0.42[1]	<0.0001
C18:3ω3	0.09±0.02	0.06±0.02	0.57±0.02[1]	<0.0001
C20:5ω3	0.47±0.02	0.25±0.02	4.93±0.20[1]	<0.0001
C22:5ω3	1.51±0.05	1.18±0.04	3.91±0.14[1]	<0.0001
C22:6ω3	4.18±0.13	3.22±0.09	6.49±0.18[1]	<0.0001

Mean ± SEM; [1] p<0.0001 vs. PO and SO.

Table 5: Fatty acid composition of mitochondrial phospholipids in myocardial cells (expressed as % of total fatty acids).

	PO (n=16)	SO (n=14)	MED (n=16)	P
Phosphatidylcholine				
Total saturated	45.0±0.91	45.0±1.27	41.6±0.69[1]	<0.05
Total ω6	40.7±0.97[2]	38.8±1.18	38.0±0.60	0.15
C18:2ω6	17.6±0.61	20.11±0.64	21.2±0.75	<0.005
C20:4ω6	21.9±0.71	17.5±0.69	15.7±0.89[3]	<0.0001
Total ω 3	3.51±0.20	3.64±0.13	11.2±0.40[3]	<0.0001
C18:3ω3	0.02±0.01	0.07±0.01	0.41±0.03[3]	<0.0001
C20:5ω3	0.17±0.02	0.10±0.01	1.13±0.06[3]	<0.0001
C22:5ω3	0.77±0.06	0.82±0.04	2.13±0.09[3]	<0.0001
C22:6ω3	2.55±0.14	2.65±0.11	7.49±0.34[3]	<0.0001
Phosphatidylethanolamine				
Total saturated	44.1±1.11[2]	36.3±0.13	38.8±1.08	<0.0001
Total ω6	31.3±0.84	36.1±1.01[4]	21.6±0.73	<0.0001
C18:2ω6	8.87±0.51	12.1±0.63	8.93±0.59	<0.005
C20:4ω6	20.4±0.55	22.0±0.45	11.7±0.29[3]	<0.0001
Total ω3	16.6±0.62	19.6±0.96	34.0±1.70[3]	<0.0001
C18:3ω3	0.01±0.01	0.06±0.01	0.46±0.03[3]	<0.0001
C20:5ω3	0.20±0.01	0.18±0.015	1.24±0.05[3]	<0.0001
C22:5ω3	1.41±0.1	1.68±0.07	2.70±0.13[3]	<0.0001
C22:6ω3	15.0±0.54	17.6±0.90	29.6±1.59[3]	<0.0001
Cardiolipin				
Total saturated	11.95±0.42[2]	5.54±0.26	5.58±11.80	<0.0001
Total ω6	81.13±0.62[2]	89.24±0.40	86.63±0.28	<0.0001
C18:2ω6	78.64±0.60[2]	87.81±0.40	84.86±0.31	<0.0001
C20:4ω6	0.72±0.02[2]	0.59±0.02	0.54±0.02	<0.0001
Total n ω3	0.97±0.03	0.69±0.05	2.86±0.17[3]	<0.0001
C18:3ω3	0.21±0.01	0.15±0.01	1.53±0.12[3]	<0.0001
C20:5ω3	0.08±0.01	0.06±0.01	0.34±0.01[3]	<0.0001
C22:5ω3	0.25±0.007	0.21±0.01	0.45±0.03[3]	<0.0001
C22:6ω3	0.43±0.02	0.28±0.02[4]	0.54±0.04	<0.0001

Mean ± SEM; [1] $p<0.05$ MED vs. PO and SO, [2] $p<0.0001$ vs. SO and MED, [3] $p<0.0001$ vs. PO and SO, [4] $p<0.0001$ vs. PO and MED.

Table 6: Left ventricular function in the 3 experimental groups.

[A]: after 15-min stabilization (baseline)

	PO (n=13)	SO (n=10)	MED (n=11)	P
Coronary flow (ml/min)	15.3± 0.3	15.1± 0.5	14.1± 0.4	NS
Diastolic pressure (mmHg)	4.2±0.1	3.9±0.1	4.4±0.1	NS
LVDevP (mmHg)	140±9	132±10	124±7.1	NS
+dP/dt (mmHg/s)	3545±106	3462±150	3313±148	NS
-dP/dt (mmHg/s)	2122±101	2198±152	2100±97	NS

[B]: after 30-min ischemia and 30-min reperfusion

	PO	SO	MED	P
Coronary flow (% baseline)	79.9 ±1.4	81.5 ±2.5	80.8 ±3.0	NS
Diastolic pressure (mmHg)	27.7 ±1.4	26.6±2.5	25.6 ±2.2	NS
LVDevP (% baseline)	63.7±2.5	66.3±30	70.2±2.5	NS
+dP/dt (% baseline)	61.7±3.1	71.8±4.4	71.4±3.4	NS
-dP/dt (% baseline)	75.8±3.7	79.2±3.6	**77.7±3.3**	NS

[C]: after 120-min reperfusion

	PO	SO	MED	P
Coronary flow (% baseline)	58.4±2.1	63.6±2.7	58.1±3.4	NS
Diastolic pressure (mmHg)	36.9±1.5	33.8±3.4	36.2 ±2.4	NS
LVDevP (% baseline)	46.3±1.9	46.5±3.9	46.4±2.4	NS
+dP/dt (% baseline)	47.5±2.6	53.1±3.5	53.5±2.8	NS
-dP/dt (% baseline)	54.0±4.3	59.0±2.4	55.5±4.1	NS

Figure 1: Effect of 8-week fat supplementation on body weight in the 3 groups

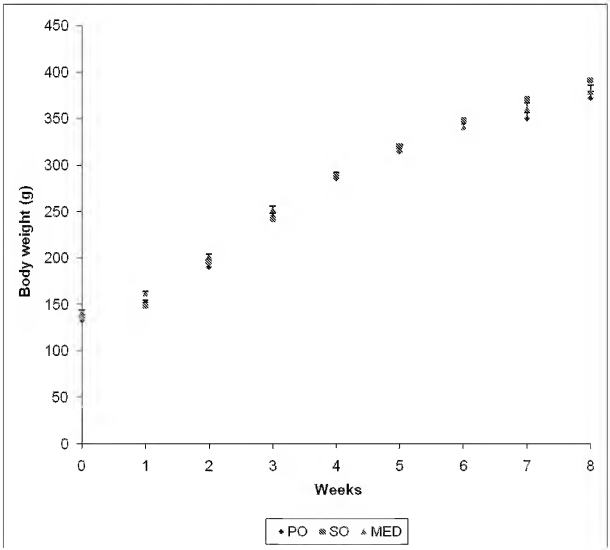

Figure 2: Risk zone (% total LV volume) and infarct size (% risk zone) in the 3 experimental groups. ANOVA P: non significant for risk zone and $p<0.01$ for infarct size.